

(19)

Europäisches Patentamt

European Patent Office

Office européen des brevets



(11)

EP 0 745 671 A2

(12)

EUROPEAN PATENT APPLICATION

(43) Date of publication:

04.12.1996 Bulletin 1996/49

(51) Int. Cl.⁶: C12N 15/00, C12N 15/61,
C12P 13/22

(21) Application number: 96105800.5

(22) Date of filing: 02.12.1991

(84) Designated Contracting States:
BE DE FR GB IT(30) Priority: 30.11.1990 JP 337221/90
02.05.1991 JP 196226/91(62) Application number of the earlier application in
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(54) **Recombinant DNA sequences encoding feedback inhibition released enzymes, plasmids comprising the recombinant DNA sequences, transformed microorganisms useful in the production of aromatic amino acids, and a process for preparing aromatic amino acids by fermentation**

(57) A method for releasing feedback inhibition of the key enzymes in the production of aromatic amino acids by fermentation is disclosed. Aromatic amino acids are prepared by a process which comprises transforming a microorganism with a recombinant DNA sequence bearing a gene or gene group encoding a feedback inhibition-released enzyme in the phenylalanine and/or tryptophan biosynthetic pathway, obtained by substituting one or two amino acid residue(s) or deleting one or more amino acid residue(s) of 3-deoxy-D-arabinoheptulonic acid 7-phosphate synthase (DS) or prephenate dehydratase, culturing the microorganism and isolating the aromatic amino acid produced in the medium. Higher efficiency and improved yields in the production of L-phenylalanine and L-tryptophan by fermentation are realized.

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Description

BACKGROUND OF THE INVENTION

5 Field of the Invention:

The present invention relates to recombinant DNA sequences encoding feedback inhibition released enzymes, plasmids containing these recombinant DNA sequences, microorganisms transformed with these plasmids, and a process for preparing L-tryptophan, L-phenylalanine and L-tyrosine by fermentation.

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Discussion of the Background:

Demand for aromatic amino acids is rapidly increasing. For example, L-phenylalanine is used as a raw material for the sweetener aspartame, L-tryptophan is an important feed additive, and all three (L-phenylalanine, L-tryptophan, and L-tyrosine) are useful as transfusion drugs.

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Many methods for preparing aromatic amino acids using microorganisms are known. For example, methods for preparing L-phenylalanine using recombinant *Escherichia coli* are described in Japanese Published Unexamined Patent Application Nos. 56-1890, 58-103398, 61-92565 and 1-104160, and World Patent Publication WO 87/00202. A method for preparing L-phenylalanine or L-tyrosine using a mutant belonging to *Coryneform* bacteria is described in Japanese Patent Published Unexamined Application No. 61-128897, and methods using recombinant *Coryneform* bacteria are described in Japanese Unexamined Published Patent Application Nos. 60-34197, 60-24192, 61-260892 and 61-124375. A method for preparing L-tryptophan using recombinant *E. coli* is described in Japanese Published Unexamined Patent Application No. 57-71397 and U.S. Patent No. 4,371,614; methods using mutants of *Bacillus subtilis* are described in Japanese Published Unexamined Patent Application Nos. 53-39517 and 62-34399; methods use recombinant *Bacillus subtilis* are described in Japanese Patent Published Unexamined Application Nos. 61-104790 and 62-34399; methods using a mutant of *Coryneform* bacteria are described in Japanese Published Unexamined Patent Application No. 57-174096; and a method using recombinant *Coryneform* bacteria is described in Japanese Published Unexamined Patent Application. No. 62-51980.

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Generally, in the biosynthetic route of aromatic amino acids, a key enzyme which plays a central role in the biosynthesis is subject to feedback inhibition by the final product. In the methods described above, the desired amino acids are principally produced using microorganisms wherein the key enzyme is released from feedback inhibition by the final product. The key enzymes released from feedback inhibition in the above methods include 3-deoxy-D-arabinoheptulonic acid-7-phosphate synthase (hereafter abbreviated as "DS") and prephenate dehydratase (hereafter abbreviated as "PD").

25

Turning first to DS, among the microorganisms used in the methods described above, *Escherichia coli* has three types of naturally-occurring (wild-type) DS isozymes. These isozymes are encoded by genes called *aroF*, *aroG* and *aroH*, which are subject to feedback inhibition by L-tyrosine, L-phenylalanine and L-tryptophan, respectively.

30

The nucleotide sequences and amino acid sequences relevant to these genes and enzymes are already reported [*aroF*: Hudson, G.S. and Davidson, B.E., J. Mol. Biol., **180**, 1023 (1984); *aroG*: Davies, W.D. and Davidson, B.E., Nucleic Acids Res., **13**, 4045 (1982); *aroH*: Ray, J.M. et al., J. Bacteriol., **170**, 5500 (1988)].

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In order to efficiently produce the desired aromatic amino acids, expression of these DS genes must be improved. With respect to *aroH*-encoded DS, feedback inhibition by L-tryptophan has been released using mutant *aroH* [Ray, J.M. et al., J. Bacteriol., **170**, 5500 (1988)]. However, the DS activity derived from *aroH* is very poor, and the *aroH*-derived DS is unsuitable for improvement by recombinant DNA techniques. It is more efficient to utilize *aroF*- or *aroG*-encoded DS in which feedback inhibition is released ("feedback inhibition-released" DS).

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An example of a mutation which releases feedback inhibition of *aroF*-encoded DS by L-tyrosine is the substitution of the 148 proline residue from the N-terminus (¹⁴⁸Pro) with a leucine residue [Weaver, L.M. and Herrmann, K.M., J. Bacteriol., **172**, 6581 (1980)].

45

Only a few examples as shown below for the production of aromatic amino acids by fermentation employ feedback inhibition-released DS with a clearly shown mutation site. Edwards et al. teach that feedback inhibition by L-tyrosine in the production of L-phenylalanine by fermentation is released by substituting the 152 glutamine residue (¹⁵²Gln) of DS encoded by *aroF* with isoleucine [WO 87/00202]. Furthermore, Sinenki et al. teach that feedback inhibition by L-phenylalanine in the production of L-phenylalanine by fermentation is suppressed by substituting the 76 leucine residue (⁷⁶Leu) of DS encoded by *aroG* with valine [Japanese Published Unexamined Patent Application No. 58-103398]. However, the enzyme activity of the feedback inhibition-released DS and the amount of L-phenylalanine produced are unknown. No reports of the production of L-tryptophan by feedback inhibition-released DS mutants are known.

50

Turning next to PD, a wild-type bifunctional enzyme (CM-PD) present in *Escherichia coli* having both chorismate mutase (hereafter abbreviated as "CM") activity and PD activity is subject to feedback inhibition by L-phenylalanine. The enzyme is encoded by a gene called *pheA*. The nucleotide sequence of *pheA* and the amino acid sequence of wild-type

CM-PD are known [Hudson, G.S. and Davidson, B.E., J. Mol. Biol., 180, 1023 (1984)]. In order to efficiently produce L-phenylalanine, it is important to release the feedback inhibition of CM-PD by L-phenylalanine.

Some examples of modification and mutation on an amino acid level are known to release feedback inhibition for the fermentative production of L-phenylalanine. By modifying two tryptophane residues (226 and 338 amino acids from the N-terminus) of CM-PD with dimethyl(2-hydroxy-5-nitrobenzylsulfonium bromide), an enzyme having resistance to feedback inhibition can be obtained [Gething, M.J.H. and Davidson, B.E., Eur. J. Biochem., 78, 111 (1977)]. Feedback inhibition-released enzyme can be obtained by deleting the 338 tryptophan residue (³³⁸Trp) or substituting ³³⁸Trp and the subsequent residues with arginine-glycine (Japanese Published Unexamined Patent Application No. 1-235597). Inserting the amino acid sequence tryptophan-arginine-serine-proline into the site of the same 338 tryptophan residue also releases feedback inhibition (WO 87/00202). These techniques focus on the 338 tryptophan residue. However, no definitive study on the effects of modifying or mutating the ²²⁶Trp residue has been performed.

On the other hand, in Corynebacterium bacteria, PD is subject to feedback inhibition by L-phenylalanine. A gene in which the feedback inhibition by L-phenylalanine has been released is known. [Ozaki, A. et al., Agric. Biol. Chem., 49, 2925 (1986); Ito, H. et al., Appl. Microbiol. Biotechnol., 33, 190 (1989)]. The nucleotide sequence of the wild type Corynebacterium PD gene shows homology to the pheA gene of Escherichia coli K-12 [Follettie, M.T. and Sinsky, A.J., J. Bacteriol., 167, 695 (1986)]. However, the nucleotide sequence of the feedback inhibition-released PD gene in Corynebacterium bacteria is unknown, as is mutation of the nucleotide sequence and release of feedback inhibition by substitution of the corresponding amino acid sequence.

20 SUMMARY OF THE INVENTION

Accordingly, one object of the present invention is to provide a process for efficiently preparing an aromatic amino acids by fermentation.

A further object is to provide transformed microorganisms useful in the production of aromatic amino acids by fermentation.

A further object is to provide recombinant plasmids which express genes encoding key enzymes in the biosynthesis of aromatic amino acids in which feedback inhibition is released.

A further object is to provide recombinant DNA sequences which encode key enzymes in the biosynthesis of aromatic amino acids in which feedback inhibition is released.

A further object is to provide novel recombinant enzymes which are important in the biosynthesis of aromatic amino acids in which feedback inhibition is released.

These and other objects which will become apparent during the following detailed description of the preferred embodiments have been accomplished by a recombinant DNA sequence encoding an enzyme of the aromatic amino acid biosynthetic pathway, wherein feedback inhibition is released, a plasmid comprising the recombinant DNA sequence, a microorganisms useful in the production of aromatic amino acids transformed with one or more of the plasmids, and a process for preparing an aromatic amino acid which comprises culturing the transformed microorganism and isolating the aromatic amino acid produced thereby.

40 BRIEF DESCRIPTION OF THE DRAWINGS

A more complete appreciation of the invention and many of the attendant advantages thereof will be readily obtained as the same becomes better understood by reference to the following detailed description when considered in connection with the accompanying drawings, wherein:

- 45 Fig. 1 shows the construction of the plasmids pTS-aroF and pTS-aroG;
- Fig. 2 shows the extent of inhibition by L-tyrosine of activity in DS encoded by both wild-type and mutant aroF;
- Fig. 3 shows the extent of inhibition by L-phenylalanine of activity in DS encoded by both wild type and mutant aroG;
- Fig. 4 shows the extent of inhibition by L-phenylalanine in the prephenate dehydratase activity of both wild-type and mutant chorismate mutase-prephenate dehydratase;
- 50 Fig. 5 shows the construction of the plasmid pACKG4.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

55 The present invention relates to a mutant feedback inhibition-released enzyme in the biosynthetic pathway of aromatic amino acids; a recombinant DNA sequence encoding an enzyme of the aromatic amino acid biosynthetic pathway, wherein feedback inhibition is released; a plasmid comprising a recombinant DNA sequence encoding an enzyme of the aromatic amino acid biosynthetic pathway, wherein feedback inhibition is released; a microorganism useful in the production of aromatic amino acids transformed with one or more plasmids comprising a recombinant DNA sequence

encoding an enzyme of the aromatic amino acid biosynthetic pathway, wherein feedback inhibition is released; and a process for preparing an aromatic amino acid which comprises culturing a microorganism transformed with one or more plasmids comprising a recombinant DNA sequence encoding an enzyme of the aromatic amino acid biosynthetic pathway, wherein feedback inhibition is released, and isolating the aromatic amino acid produced thereby.

5 In the present application, the phrase "aromatic amino acid" refers to L-phenylalanine, L-tryptophan and L-tyrosine. Also, an enzyme is "released" from feedback inhibition by a final product if the activity of the enzyme doesn't decrease in the presence of the final product.

Preferably, the enzymes of the present invention in the biosynthetic pathway of aromatic amino acids which are to be released from feedback inhibition are 3-deoxy-D-arabinoheptulonic acid-7-phosphate synthase (DS), prephenate
10 dehydratase (PD) and chorismate mutase-prephenate dehydratase (CM-PD). The means by which each of these enzymes is released from feedback inhibition in the present invention is preferably by mutation, wherein one or two amino acid residue(s) are substituted with other amino acid residue(s) or one or more amino acid residue(s) are deleted. Furthermore, the transformed microorganism preferably belongs to the genus *Escherichia*, and is preferably transformed with one or more plasmids bearing a recombinant DNA sequence corresponding to one of the above
15 mutant enzymes.

Firstly, the present inventors have acquired a novel gene encoding DS, wherein the feedback inhibition is released by cloning the natural DS gene of *Escherichia coli* and subjecting the cloned gene to mutation. Furthermore, the natural PD gene of *Brevibacterium lactofermentum* is cloned, and a gene encoding PD in which feedback inhibition is released, is cloned from L-phenylalanine-producing *Corynebacterium* bacteria. Even further, the natural CM-PD gene of *Escherichia*
20 *coli* is cloned, and the cloned gene is then subjected to mutation to produce a novel gene encoding CM-PD in which feedback inhibition is released. By transfecting or transforming phenylalanine-producing bacteria with one or two of the genes of the present invention, production of L-phenylalanine by fermentation can be improved.

The present inventors have also improved the fermentative production of L-tryptophan by transforming a microorganism with the novel DS gene of the present invention in combination with a tryptophan operon, in which the feedback
25 inhibition of anthranilate synthase (hereafter abbreviated as AS), an enzyme for the L-tryptophan biosynthesis system, is also released.

The novel gene of the present invention encoding DS having released feedback inhibition is prepared by the following procedure.

Firstly, *aroF* and *aroG* genes are cloned from chromosomal DNA of *Escherichia coli* using the PCR method as
30 described in U.S. Patent Nos. 4,800,159, 4,683,202 and 4,683,195, all incorporated herein by reference. The chromosomal DNA appropriate as a source of the *aroF* and *aroG* genes for use in the present invention may be cloned from any strain of *Escherichia coli*, but the preferred strain is K-12 MC1061 (ATCC 53338). The desired genes are then mutated with hydroxylamine by a known method; for example, that described in J. Mol. Biol., 175, 331 (1984).

The genes *aroF* and *aroG* encode DS which is subject to feedback inhibition by L-tyrosine and L-phenylalanine,
35 respectively, and also include mutants caused by genetic polymorphism, etc. Genetic polymorphism refers to a modification of an amino acid sequence of a protein due to natural mutation of a gene.

In order to cause mutation of the gene, a number of effective methods are known. Examples include recombinant PCR methods [PCR Technology, Stockton Press (1989)], site specific mutation [Kramer, W. and Frits, H.J., Methods in Enzymology, 154, 350 (1987)], conventional methods exposing a strain bearing the gene to UV (ultraviolet light) rays,
40 conventional methods treating the DNA or DNA-bearing microorganism with a chemical (N-methyl-N'-nitrosoguanidine, nitric acid, etc.), and conventional methods for chemical synthesis of the desired gene, such as those employing a known automated synthesizer.

In the present invention, the mutated amino acid residue of DS is in the region of the amino acid sequence which participates in the mechanism of feedback inhibition by L-tyrosine, L-phenylalanine or L-tryptophan. For example, in DS
45 encoded by *aroF*, the 147 aspartic acid residue (¹⁴⁷Asp) and the 181 serine residue (¹⁸¹Ser) from the N-terminus are the mutated amino acid residues. Any mutation of the amino acid residue which results in release from feedback inhibition is suitable. For example, substitution, deletion, or addition is suitable. The DS mutations and the corresponding nucleotide sequence mutations exemplified in the present invention are summarized in Table 1.

By transfecting a suitable microorganism with the mutant *aroF* or *aroG* gene above as a recombinant DNA
50 sequence, the microorganism can express the recombinant mutant gene in which feedback inhibition is released.

In the present invention, the novel gene encoding PD in *Brevibacterium lactofermentum* and the novel gene encoding CM-PD in *Escherichia coli* were prepared as follows.

Firstly, the nucleotide sequence of the *Brevibacterium lactofermentum* PD gene encoding PD in which feedback inhibition by L-phenylalanine is released was determined and analyzed. It has thus been found that the L-phenylalanine-producing strain expressed PD in which one amino acid is substituted, as compared to the wild strain. Next,
55 based on this finding, a substitution or a deletion of amino acid residue(s) was carried out at the corresponding position of CM-PD in *Escherichia coli* K-12, resulting in CM-PD in which the feedback inhibition is released.

Enzymes having PD activity referred to in the present invention refer to enzymes derived from microorganisms such as *Corynebacterium* bacteria having PD activity, and further refer to enzymes derived from microorganisms such as

Escherichia coli, etc., having the bifunctional activity of CM-PD.

In the present invention, the mutated amino acid residue of PD refers to a substitution of an amino acid residue or a deletion of amino acid residue(s) present in the region of the amino acid sequence which participates in the mechanism of feedback inhibition by L-phenylalanine. For example, in PD derived from Brevibacterium lactofermentum, the 235 serine residue (²³⁵Ser) is suitable for mutation, and in CM-PD derived from Escherichia coli, the 330 serine residue (³³⁰Ser) from the N-terminus is an amino acid residue suitable for mutation. Suitable mutations include any which result in the release of feedback inhibition, but particularly suitable mutations include substitutions of ²³⁵Ser or ³³⁰Ser with proline or aspartic acid residue, or deletion of amino acid residues down stream from ³³⁰Ser.

By transfecting a suitable microorganism with the mutant PD or CM-PD gene described above as a recombinant DNA sequence, expression of PD in which feedback inhibition is released is achieved in the transfected microorganism.

The recombinant DNA sequences obtained by the foregoing procedures refer to those obtained by incorporating a gene encoding feedback inhibition-released DS or PD into a vector of plasmid or phage DNA. In the present invention, promoters such as lac, trp, PL, etc. which act in the microorganism may also be used to efficiently perform the expression of the gene. The recombinant DNA sequences referred to herein include those obtained by incorporating one or more of the above-described genes into a chromosome according to known methods. Examples include methods using a transposon (Berg, D.E. and Berg, C.M., Bio/Technol., 1, 417 (1983)), Mu phage (Japanese Published Unexamined Patent Application No. 2-109985) or homologous recombination [Experiments in Molecular Genetics, Cold Spring Harbor Laboratory (1972)].

As the microorganism containing the recombinant DNA, any microorganism may be used, irrespective of species and strain of the microorganism, so long as it expresses the gene encoding the desired enzyme (such as DS or PD) and is capable of producing the aromatic amino acid (for example, in the case of L-phenylalanine, the microorganism which has acquired L-phenylalanine productivity by imparting L-phenylalanine analog resistance thereto). Particularly suitable microorganisms are selected from the genus Escherichia, the genus Brevibacterium, the genus Corynebacterium, the genus Bacillus, the genus Serratia, the genus Pseudomonas, etc.

The thus obtained microorganism transformed by the recombinant DNA bearing the feedback inhibition-released DS or PD gene is cultured, the desired aromatic amino acid is produced by the transformed microorganism in a suitable medium, and the accumulated aromatic amino acid is collected and isolated.

The medium used for producing the aromatic amino acid is a conventional medium containing appropriate carbon sources, nitrogen sources, inorganic ions and, if necessary, other organic components.

Suitable carbon sources include sugars such as glucose, lactose, galactose, fructose, starch hydrolysate, etc.; alcohols such as glycerol, sorbitol, etc.; organic acids such as fumaric acid, citric acid, succinic acid, etc.

Suitable nitrogen sources include inorganic ammonium salts such as ammonium sulfate, ammonium chloride, ammonium phosphate, etc.; organic nitrogen such as soybean hydrolysate, etc.; ammonia gas, ammonia water, etc.

Suitable organic trace nutrient sources preferably are present, and include required substances such as vitamin B₁, L-tyrosine, or yeast extract, etc., in an appropriate amount. In addition thereto, small amounts of potassium phosphate, magnesium sulfate, iron ions, manganese ions, etc. may be present.

Incubation is carried out for 16 to 72 hours under aerobic conditions. The temperature for incubation is maintained between 30 and 45°C and the pH is maintained in the range of 5 to 7 during the incubation. The pH may be adjusted with either acids or alkaline substances, which may be inorganic or organic, or may be adjusted with ammonia gas, etc., as is appropriate to maintain the desired pH and concentrations of components in the medium.

The desired aromatic amino acid is isolated from the fermentation medium generally by conventional methods, such as use of an appropriate ion exchange resin, precipitation, and/or other known techniques, either alone or in combination.

By the general process described above, the transformant expressing feedback inhibition-released DS, PD and/or CM-PD is obtained, and by culturing the transformant, the productivity of aromatic amino acids can be greatly improved.

Other features of the invention will become apparent in the course of the following descriptions of exemplary embodiments which are given for illustration of the invention, and are not intended to be limiting thereof.

Example 1: Preparation of a novel gene encoding DS in which the feedback inhibition is released

(1) Collection of an aroF-derived mutant DS gene of Escherichia coli

Chromosomal DNA was extracted from Escherichia coli K-12 MC1061 strain in a conventional manner. In a separate procedure, two synthetic DNA primers shown by Sequence Nos. 1 (SEQ ID NO:1) and 2 (SEQ ID NO:2) were synthesized in a conventional manner, based on the known nucleotide sequence of the target aroF gene [J. Mol. Biol., 180, 1023 (1984)].

Sequence No. 1 GCTAACCAGT AAAGCCAACA (SEQ ID NO:1)

Sequence No. 2 CCCACTTCAG CAACCACTTC (SEQ ID NO:2)

These primers have homologous sequences upstream and downstream from the aroF gene. Using the chromosomal DNA and the DNA primers, PCR (polymerase chain reaction) is conducted according to the method of Erlich et al. [PCR Technology, Stockton Press (1989)], yielding a DNA fragment of 1.5 Kbp. Thereafter, as shown in Fig. 1, left side, the fragment is cleaved with restriction enzymes EcoRV and Eco47III, and the product is then ligated with the SmaI digestion product of pHSG398 (manufactured by Takara Shuzo) using T4 DNA ligase. Competent cells of Escherichia coli JM109 strain (manufactured by Takara Shuzo) were transformed with the reaction mixture. A plasmid having the aroF gene was extracted from the strains resistant to chloramphenicol to yield the plasmid pHSG-aroF.

Subsequently, pHSG-aroF was digested with restriction enzymes EcoRI and HindIII, and the resulting DNA fragment bearing the aroF gene was ligated with the EcoRI and HindIII digestion fragment of plasmid pTS1 (Japanese Patent Application No. 2-192162) using T4 DNA ligase. Competent cells of DS-deleted (aroF, aroG, aroH) strain AB3257 of Escherichia coli K-12 were transformed with the reaction mixture (AB3257 strain was acquired from the Escherichia coli Genetic Stock Center). From among the strains resistant to ampicillin, the strain in which auxotrophy of L-tyrosine, L-phenylalanine and L-tryptophan disappeared was selected, and a plasmid was extracted therefrom, yielding plasmid pTS-aroF.

Next, after mutation of plasmid pTS-aroF using hydroxylamine according to the method of J. Mol. Biol., 175, 331 (1984), the mutant was used to transform the E. coli AB3257 strain. After ampicillin-resistant strains were collected, two strains which grew in minimum medium supplemented with 1 mM L-tyrosine were selected. From these strains, plasmids pTS-aroF15 and pTS-aroF33 bearing the genes encoding feedback inhibition-released DS were obtained.

Cells of AB3257 strain transformed with plasmids containing the gene encoding non-feedback inhibition-released DS are subject to feedback inhibition at 1 mM concentration of L-tyrosine in the minimum medium. Accordingly, the strain subject to feedback inhibition failed to synthesize aromatic amino acids such as L-phenylalanine or L-tryptophan, and failed to grow.

(2) Preparation of aroG-derived mutant DS gene of Escherichia coli

A mutant aroG gene was collected in a manner similar to the case of the aroF gene. Two synthetic DNA primers shown by Sequence Nos. 3 (SEQ ID NO:3) and 4 (SEQ ID NO:4) were synthesized in a conventional manner, based on the known nucleotide sequence of the aroG gene (Nucleic Acids Res., 10, 4045 (1982)).

Sequence No. 3 GTATTTACCC CGTTATTGTC (SEQ ID NO:3)

Sequence No. 4 ACTCCGCCGG AAGTGACTAA (SEQ ID NO:4)

Using the primers and the chromosomal DNA of the E. coli MC1061 strain, PCR was carried out to obtain a DNA fragment of 2.1 Kbp. As shown in Fig. 1, right side, the fragment was cleaved with restriction enzymes Sall and Eco47III, and the product was then ligated with the Sall and SmaI digestion product of pHSG398 (manufactured by Takara Shuzo) using T4 DNA ligase. Competent cells of Escherichia coli JM109 strain were transformed with the reaction mixture. From among the strains resistant to chloramphenicol, a plasmid having the aroG gene was extracted to yield the plasmid pHSG-aroG.

Subsequently, pHSG-aroG was digested with restriction enzymes EcoRI and HindIII, and the resulting DNA fragment bearing the aroG gene was ligated with the EcoRI and HindIII digestion fragment of plasmid pTS1 using T4 DNA ligase. From among the grown strains resistant to ampicillin, the strain in which auxotrophy of L-tyrosine, L-phenylalanine and L-tryptophan disappeared was selected, and a plasmid was extracted therefrom to yield the plasmid pTS-aroG.

Next, after mutation of the plasmid using the hydroxylamine method of Example 1-(1) above, the mutant plasmid was used to transform competent cells of the E. coli AB3257 strain. After ampicillin-resistant strains were isolated, 6 strains which grew in minimum medium supplemented with 10 mM L-phenylalanine were selected. From these strains, plasmids pTS-aroG4, pTS-aroG8, pTS-aroG15, pTS-aroG17, pTS-aroG29 and pTS-aroG40 bearing the aroG gene encoding feedback inhibition-released DS were obtained.

In cells of the AB3257 strain expressing the non-feedback inhibition-released DS, feedback inhibition occurs at a concentration of 10 mM L-phenylalanine in minimum medium. Accordingly, the non-feedback inhibition-suppressed strain fails to synthesize aromatic amino acids such as L-tryptophan and/or L-tyrosine, and therefore, fails to grow.

(3) Determination of DS enzyme activity

The above plasmids, bearing either mutant aroF (pTS-aroF15 and pTS-aroF33) or mutant aroG (pTS-aroG4, pTS-aroG8, pTS-aroG15, pTS-aroG17, pTS-aroG29 and pTS-aroG40), were used to transform Escherichia coli AB3257 strain having no DS activity. The respective transformants were named AJ 12598 (AB3257/pTS-aroF15), AJ 12599

(AB3257/pTS-aroF33), AJ 12562 (AB3257/pTS-aroG4), AJ 12600 (AB3257/pTS-aroG8), AJ 12563 (AB3257/pTS-aroG15), AJ 12601 (AB3257/pTS-aroG17), AJ 12602 (AB3257/pTS-aroG29) and AJ 12603 (AB3257/pTS-aroG40), respectively. Among them, AJ 12563 and AJ 12603 were deposited as representative strains in the Fermentation Research Institute of the Agency of Industrial Science & Technology of Japan, under the deposit numbers Escherichia coli FERM BP-3567 and FERM BP-3568, respectively. For the purpose of comparison, plasmids bearing wild type genes were also used to transform the E. coli AB3257 strain.

Each of these strains were cultured for 24 hours in a known L-phenylalanine-producing medium [Sugimoto, S. et al., J. Biotechnol., 5, 237 (1988)]. From the culture cells, the crude enzyme solution was prepared by ultrasonic homogenization. The enzyme activity of DS was determined in a conventional manner [Gollub, E. et al., Methods Enzymol., 17, 349], in the presence of L-tyrosine in the case of aroE, and in the presence of L-phenylalanine in the case of aroG. The results presented in Figs. 2 and 3 show that the DS enzyme activity of the wild type transformants (Escherichia coli AB3257/pTS-aroF) is strongly inhibited in the presence of L-tyrosine, whereas the respective mutant transformants are released from feedback inhibition by L-tyrosine. Likewise, in the wild type transformant Escherichia coli AB3257/pTS-aroG, the enzyme activity is strongly inhibited in the presence of L-phenylalanine, whereas in the respective mutant transformants, feedback inhibition by L-phenylalanine is released. Furthermore, the mutant strain AJ 12562 not only releases feedback inhibition by L-phenylalanine, but surprisingly, the DS enzyme activity increases as the concentration of L-phenylalanine increases.

(4) Determination of the mutation site of DS in which the feedback inhibition is released

The nucleotide sequences of the feedback inhibition-released aroF15, aroF33, aroG4, aroG8, aroG15, aroG17, aroG29 and aroG40 were determined in a conventional manner [Molecular Cloning (Second Edition), Cold Spring Harbor Press (1989)]. The specific substitution site on the amino acid sequence and the mutation site on the corresponding nucleotide sequence are shown in Table 1.

These sequences are all novel.

Table 1

Mutant Gene	Substitution Site of Amino Acid		Corresponding Nucleotide Sequence Change
	Position from N-Terminus	Amino Acid Sequence Change	
<u>aroF15</u>	147	Asp → Asn	GAT → AAT
<u>aroF33</u>	181	Ser → Phe	TCC → TTC
<u>aroG4</u>	150	Pro → Leu	CCA → CTA
<u>aroG8</u>	202	Ala → Thr	GCC → ACC
<u>aroG15</u>	146	Asp → Asn	GAT → AAT
<u>aroG17</u>	147	Met → Ile	ATG → ATA
	332	Glu → Lys	GAA → AAA
<u>aroG29</u>	147	Met → Ile	ATG → ATA
<u>aroG40</u>	157	Met → Ile	ATG → ATA
	219	Ala → Thr	GCG → ACG

Example 2: Preparation of a novel gene encoding PD in which the feedback inhibition is released

(1) Determination of the mutation site of Brevibacterium lactofermentum mutant PD

The nucleotide sequence of the NcoI fragment in plasmid pAJ16 bearing the PD gene of Brevibacterium lactofermentum wild strain was determined by the dideoxy method, using the homology to known Corynebacterium s.p. PD gene [Follettie, M.T. and Sinsky, A.J., J. Bacteriol., 167, 695 (1986)] as an index. The plasmid is harbored on Brevibacterium lactofermentum AJ 12125 (FERM P-7546). The resulting nucleotide sequence (SEQ ID NO:5) and the corresponding amino acid sequence (SEQ ID NO:6) are shown below. The B. lactofermentum PD amino acid sequence is

different by only one amino acid residue from that of Corynebacterium s.p.

Sequence 5:

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5  ATGAGCGACGCACCAATTGTTGTGGCCTATTTGGGGCCTGCCGGAACCTTCACCGAAGAA
   MetSerAspAlaProIleValValAlaTyrLeuGlyProAlaGlyThrPheThrGluGlu
   1                                     20

10 GCCCTCTACAAATTTGCCGACGCGGCGTATTCCGGCGACGGTGAGATCGAGCAGCTACCA
   AlaLeuTyrLysPheAlaAspAlaGlyValPheGlyAspGlyGluIleGluGlnLeuPro
   21                                     40

15 GCCAAATCGCCACAAGAAGCTGTCGACGCGGTCCGCCACGGCACCGCCCAGTTCGCGGTG
   AlaLysSerProGlnGluAlaValAspAlaValArgHisGlyThrAlaGlnPheAlaVal
   41                                     60

20 GTCGCCATCGAAAACCTTCGTCGACGGCCCCGTCACCCCCACCTTCGACGCCCTTGACCAG
   ValAlaIleGluAsnPheValAspGlyProValThrProThrPheAspAlaLeuAspGln
   61                                     80

25 GGCTCCAACGTGCAAATCATCGCCGAAGAAGAACTCGATATTGCCTTTTCCATCATGGTC
   GlySerAsnValGlnIleIleAlaGluGluGluLeuAspIleAlaPheSerIleMetVal
   81                                     100

```


5 CGGCCAGGGACTTCGCTTGCCGACGTCAAACCCTCGCCACCCACCGGTTGGGTACCAA
 ArgProGlyThrSerLeuAlaAspValLysThrLeuAlaThrHisProValGlyTyrGln
 101 120
 CAAGTGAAAACTGGATGGCAACCACCATTCGGGACGCCATGTATCTTTCAGCAAGCTCC
 GlnValLysAsnTrpMetAlaThrThrIleProAspAlaMetTyrLeuSerAlaSerSer
 121 140
 10 AACGGCGCCGGCGCACAAATGGTTGCCGAAGGAACGGCCGACGCGAGCGCGAGCGCCCTCC
 AsnGlyAlaGlyAlaGlnMetValAlaGluGlyThrAlaAspAlaAlaAlaProSer
 141 160
 CGCGCAGCCGAACTCTTCGGAAGCGCCTTGTTGATGATGTGCGCCGACGTCCGCGGC
 ArgAlaAlaGluLeuPheGlyLeuGluArgLeuValAspAspValAlaAspValArgGly
 15 161 180
 GCCCGCACCCGCTTCGTTGCAGTCCAAGCCCAAGCAGCCGTTTCCGAACCGACCGCCAC
 AlaArgThrArgPheValAlaValGlnAlaGlnAlaAlaValSerGluProThrGlyHis
 20 181 200
 GACCGCACCTCCGTCATTTCTCCCTACCGAATGTGCCAGGCAGCCTCGTGCGCGCCCTC
 AspArgThrSerValIlePheSerLeuProAsnValProGlySerLeuValArgAlaLeu
 201 220
 25 AACGAATTCGCCATCCGTGGCGTCGACCTCACCCGCATCGAATCCCGCCCCACCGCAAA
 AsnGluPheAlaIleArgGlyValAspLeuThrArgIleGluSerArgProThrArgLys
 221 240
 GTCTTCGGAACTACCGCTTCCACCTGGACATATCCGGACATATCCGCGACATCCCGTC
 30 ValPheGlyThrTyrArgPheHisLeuAspIleSerGlyHisIleArgAspIleProVal
 241 260
 GCCGAAGCCCTCCGCGCACTCCACCTCCAAGCCGAAGAACTCGTATTCGTGCGTTCTCGG
 AlaGluAlaLeuArgAlaLeuHisLeuGlnAlaGluGluLeuValPheValGlySerTrp
 35 261 280
 CCTCCAACCGTGCAGAAGACAGCAGCCCCAAACCGACCACTAGCTAACGTACACAAG
 ProSerAsnArgAlaGluAspSerThrProGlnThrAspGlnLeuAlaAsnValHisLys
 281 300
 40 GCGGACGAATGGGTTGCGCGCAGCAAGCGAAGGAAGGAACTTAAGTAG
 AlaAspGluTrpValArgAlaAlaSerGluGlyArgLysLeuAsn***
 301 315

45 Next, the nucleotide sequence (SEQ ID NO: 7) of the gene on plasmid pPH14 encoding PD of the phenylalanine-
 producing strain of *Brevibacterium lactofermentum* was determined. The sequence shown below was obtained. The
 plasmid used was the one borne on *Brevibacterium lactofermentum* AJ 12259 (FERM BP-3565). A comparison of the
 50 amino acid sequences was made between the wild-type PD and the feedback inhibition-released PD (SEQ ID NO: 8)
 and it was found that ²³⁵Ser residue of the wild strain was mutated to a proline residue in the feedback inhibition-
 released PD.

Sequence of Brevibacterium lactofermentum PD (pPH14):

5 ATGAGCGACGCACCAATTGTTGTGGCCTATTGGGGCCTGCCGGAACCTTCACCGAAGAA
 MetSerAspAlaProIleValValAlaTyrLeuGlyProAlaGlyThrPheThrGluGlu
 1 20
 10 GCCCTCTACAAATTTGCCGACGCCGGCGTATTGGGCGACGGTGAGATCGAGCAGCTACCA
 AlaLeuTyrLysPheAlaAspAlaGlyValPheGlyAspGlyGluIleGluGlnLeuPro
 21 40
 15 GCCAAATCGCCACAAGAAGCTGTGACGCCGGTCCGCCACGGCACCGCCCAGTTCGCGGTG
 AlaLysSerProGlnGluAlaValAspAlaValArgHisGlyThrAlaGlnPheAlaVal
 41 60
 20 GTGCCATCGAAAACCTTCGTGACGCCGCCCGTCACCCCCACCTTCGACGCCCTTGACCAG
 ValAlaIleGluAsnPheValAspGlyProValThrProThrPheAspAlaLeuAspGln
 61 80
 25 GGCTCCAACGTGCAAATCATCGCCGAAGAAGAACTCGATATTGCCTTTTCCATCATGGTC
 GlySerAsnValGlnIleIleAlaGluGluGluLeuAspIleAlaPheSerIleMetVal
 81 100
 30 CGGCCAGGGACTTCGCTTGCCGACGTCAAACCCCTCGCCACCCACCGGTTGGGTACCAA
 ArgProGlyThrSerLeuAlaAspValLysThrLeuAlaThrHisProValGlyTyrGln
 101 120
 35 CAAGTGAAAAACTGGATGGCAACCACCATTCCGGACGCCATGTATCTTTCAGCAAGCTCC
 GlnValLysAsnTrpMetAlaThrThrIleProAspAlaMetTyrLeuSerAlaSerSer
 121 140
 40 AACGGCGCCGGCGCAAAATGGTTGCCGAAGGAACCGCCGACGCAGCCGCAGCGCCCTCC
 AsnGlyAlaGlyAlaGlnMetValAlaGluGlyThrAlaAspAlaAlaAlaProSer
 141 160
 45 CGCGCAGCCGAACTCTTCGGACTGGAACGCCTTGTTGATGATGTCCCGACGTCCGCGGC
 ArgAlaAlaGluLeuPheGlyLeuGluArgLeuValAspAspValAlaAspValArgGly
 161 180
 50
 55

5 GCCCGCACCCGCTTCGTTGCAGTCCAAGCCCAAGCAGCCGTTTCCGAACCGACCGGCCAC
 AlaArgThrArgPheValAlaValGlnAlaGlnAlaAlaValSerGluProThrGlyHis
 181 200
 GACCGCACCTCCGTCATTTTCTCCCTACCGAATGTGCCAGGCAGCCTCGTGGCGGCCCTC
 AspArgThrSerValIlePheSerLeuProAsnValProGlySerLeuValArgAlaLeu
 201 220
 10 AACGAATTGCCATCCGTGGCGTGCACCTCACCGCATCGAATCCCGCCCCACCGCAAA
 AsnGluPheAlaIleArgGlyValAspLeuThrArgIleGluProArgProThrArgLys
 221 240
 15 GTCTTCGGAACCTACCGCTTCCACCTGGACATATCCGGACATATCCGCGACATCCCGTC
 ValPheGlyThrTyrArgPheHisLeuAspIleSerGlyHisIleArgAspIleProVal
 241 260
 20 GCCGAAGCCCTCCGCGCACTCCACCTCCAAGCCGAAGAACTCGTATTCGTGCGTTTCCTGG
 AlaGluAlaLeuArgAlaLeuHisLeuGlnAlaGluGluLeuValPheValGlySerTrp
 261 280
 CCCTCCAACCGTGCAGAAGACAGCACGCCCCAAACCGACCAACTAGCTAACGTACACAAG
 ProSerAsnArgAlaGluAspSerThrProGlnThrAspGlnLeuAlaAsnValHisLys
 281 300
 30 GCGGACGAATGGGTTGCGCGCAGCAAGCGAAGGAAGAACTTAACTAG
 AlaAspGluTrpValArgAlaAlaSerGluGlyArgLysLeuAsn***
 301 315

(2) Construction of a novel gene encoding a mutant CM-PD of *Escherichia coli*

Chromosomal DNA was extracted from *Escherichia coli* K-12 RRI strain in a conventional manner.
 In a separate procedure, four synthetic DNA primers (Sequence Nos. 7-10) (SEQ ID NOS: 9-12) were chemically
 synthesized in a conventional manner, based on the known nucleotide sequence of the *pheA* gene [Hudson, G.S. and
 Davidson, B.E., J. Mol. Biol., 180, 1023 (1984)].

Sequence No. 7 TCAACAAGCT GGAACGGACG (SEQ ID NO: 9)
 Sequence No. 8 CGCCATTTA CCGCCTTGAG (SEQ ID NO: 10)
 Sequence No. 9 CCGTCTGGAA CCACGCCCGA T (SEQ ID NO: 11)
 Sequence No. 10 ATCGGGCGTG ATTCCAGACG G (SEQ ID NO: 12)

Sequence Nos. 7 and 8 have homologous sequences upstream and downstream from the *pheA* gene, respectively.
 Sequence Nos. 9 and 10 are complementary to each other and have almost perfect homology to the sequence around
 the 330 serine residue (³³⁰Ser), except that one base pair is different, i.e., T (thymine base) is substituted with C (cyto-
 sine base). CM-PD of *Escherichia coli* K-12 has high homology to PD of *Brevibacterium lactofermentum*. In particular,
 the 330 serine residue from the N-terminal (³³⁰Ser) of the CM-PD of *Escherichia coli* K-12 corresponds to the 235 ser-
 ine residue (²³⁵Ser) of *Brevibacterium lactofermentum* PD. Sequence Nos 9 and 10 are synthesized in such a manner
 that the 330 serine residue becomes a proline residue.

Next, using 1 µg of the chromosomal DNA and either 300 ng of each of the primers of Sequence Nos. 7 and 10, or
 300 ng of each of the primers of Sequence Nos. 8 and 9, PCR was carried out to obtain DNA fragments of 1.3 Kbp and
 0.5 Kbp, respectively. The PCR temperature cycle of reaction at 94°C for one minute, at 50°C for 2 minutes and at 72°C
 for 3 minutes was repeated for 20 cycles using a continuous replication reaction device (Thermal Cycler, manufactured
 by Perkin Elmer Cetus Co.), according to the method of Erlich et al. [PCR Technology, Stockton Press (1989)]. These
 DNA fragments were subjected to agarose gel electrophoresis and recovered using a standard DNA recovery kit (Gene
 Clean, manufactured by Funakoshi Co.).

Separately, using these fragments and the primers of Sequence Nos. 7 and 8, PCR reaction was further carried out to obtain a DNA fragment of 1.8 Kbp. After the 1.8 Kbp fragment was digested with *Bam*HI and *Pst*I, a DNA fragment of 1.7 Kbp was recovered by agarose electrophoresis. Subsequently, the 1.7 Kbp fragment was ligated with the *Bam*HI and *Pst*I digestion product of plasmid pHSG398 (manufactured by Takara Shuzo) using T4 DNA ligase. The ligation product was used to transfect *Escherichia coli* KA197 strain (*pheA*). Among the strains resistant to chloramphenicol, the strain in which phenylalanine auxotrophy disappeared was selected, and a plasmid was recovered. The plasmid was named pHAB. Its nucleotide sequence was determined. This plasmid bears the mutant CM-PD enzyme gene in which the 330 serine residue was substituted with a proline residue.

Also using the same methods mentioned above, 330 serine residue from the N-terminal (³³⁰Ser) of the CM-PD of *Escherichia coli* K-12 was substituted with an aspartic acid residue. Sequence Nos. 11 and 12 were synthesized in such a manner that the 330 serine residue became an aspartic acid residue.

Nos. 11 CCGTCTGGAA GACCGCCCGA T
Nos. 12 ATCGGGCGGT CTTCCAGACG G

In this way, the plasmid pHAD, which bears the mutant CM-PD enzyme gene in which the 330 serine residue was substituted with an aspartic acid, was obtained.

Also using the same methods mentioned above, amino acid residues downstream from ³³⁰Ser of the CM-PD of *Escherichia coli* K-12 were deleted. Sequence Nos. 13 and 14 were synthesized in such a manner that the codon of the 330 serine residue became termination codon.

Nos. 13 CCGTCTGGAA TGACGCCCGA T
Nos. 14 ATCGGGCGTC ATTCCAGACG G

In this way, the plasmid pHATerm, which bears the mutant CM-PD enzyme gene in which the amino acid residues downstream from the 330 serine residue were deleted, was obtained.

(3) Construction of a *tyrA* gene-defected W3110 strain of *Escherichia coli* K-12

Escherichia coli W3110 strain (acquired from National Institute of Heredity) was spread on a plate medium containing streptomycin to obtain a streptomycin-resistant strain. Next, this strain was cultured in a culture medium in combination with *Escherichia coli* K-12 ME8424 strain (HfrPO45, *thi*, *relA1*, *tyrA*: : Tn10, *ung-1*, *nadB*) (acquired from National Institute of Heredity), and the medium was allowed to stand at 37°C for 15 minutes to perform conjugation transfer. Then the medium was applied to a plate medium containing streptomycin, tetracycline and L-tyrosine. The formed colony, i.e., *Escherichia coli* W3110 (*tyrA*) strain, was collected.

The plasmid pHAB, pHAD, and pHATerm obtained in Example (2)-2 above were used to transform competent cells of the *E. coli* W3110 (*tyrA*) strain. The transformant *Escherichia coli* K-12 [W3110 (*tyrA*)/pHAB] *Escherichia coli* K-12 [W3110 (*tyrA*)/pHAD], and *Escherichia coli* K-12 [W3110 (*tyrA*)/pHATerm] were deposited in the Fermentation Research Institute of the Agency of Industrial Science & Technology of Japan. The deposit numbers are FERM BP-3566, FERM BP-12659, and FERM BP-12662, respectively.

(4) Measurement of PD enzyme activity

Escherichia coli K-12 W3110 (*tyrA*)/pHAB strain was cultured at 37°C for 15 hours in L medium and the cells were collected by centrifugation. Then, the cells were washed twice with physiological saline, and suspended in 250 mM Tris-hydrochloride buffer (pH 7.5) containing 0.5 mM dithiothreitol under ice cooling. By ultrasonication (20 KHz) for 30 seconds four times, the crude enzyme solution was prepared.

The PD enzyme activity was determined in a conventional manner [Cotton, R.G.H. and Gibson, F., Meth. in Enzymol., 17, 564 (1970)]. Using the crude enzyme, the enzymatic reaction was carried out at 37°C for 10 minutes in the presence of 50 mM Tris-hydrochloride buffer (pH 8.2) containing 1 mM barium prephenate and 0.5 mM L-tyrosine. Aqueous sodium hydroxide (1 N) was added to terminate the reaction, and the formed phenylpyruvic acid was measured at an extinction wavelength of 320 nm.

Quantitative determination of protein was made using the Protein Assay Kit (manufactured by Bio Rad Co.), according to the protocol of the manufacturer.

The results presented in Fig. 4 show that the enzyme reaction in strains transformed with the wild CM-PD gene was strongly inhibited in the presence of 0.5 mM L-phenylalanine, whereas strains transformed with a mutant CM-PD gene exhibited almost no inhibition, even in the presence of 5 mM L-phenylalanine.

Further, in the case of the plasmid bearing the wild type enzyme gene, in the absence of L-phenylalanine, the enzyme activity was only 3.5×10^2 U/mg protein. In the case of the mutant CM-PD gene, the activity was 1.5×10^4 U/mg

protein. The results show that not only is expression of the mutant type CM-PD enzyme gene released from feedback inhibition by L-phenylalanine in transformants containing the mutant gene, but surprisingly, the amount of enzyme and/or enzyme activity can be increased by roughly two orders of magnitude.

By using the same method above, the PD enzyme activities of *Escherichia coli* W3110 (*tyrA*)/(pPHAD) and *Escherichia coli* W3110 (*tyrA*)/(pPHATerm) were determined. As the result, the PD enzymes of the both strains were found to be released from feedback inhibition by L-phenylalanine.

Example 3: Production, of L-phenylalanine by fermentation

- (1) Construction off *Escherichia coli* K-12 bearing a CM-PD gene alone and, in combination with a DS gene, in which the feedback inhibition is released

From pTS-*aroG4* bearing the feedback inhibition-released DS gene obtained in Example 1, the *aroG4* portion was excised with restriction enzymes *EcoRI* and *HindIII*. The fragment was inserted into the cleavage site of pBR322 with *EcoRI* and *HindIII* to obtain plasmid pBR-*aroG4* (having an ampicillin-resistant marker).

In a separate procedure, pPHAB bearing the feedback inhibition-released CM-PD gene obtained in Example 2 was digested with restriction enzymes *BamHI* and *HindIII* to excise the fragment containing the CM-PD gene. This fragment was inserted into the cleavage site of pACYC184 with *BamHI* and *HindIII* to construct plasmid pACMAB (selection marker was chloramphenicol resistance). The pACMAB plasmid was used to transform competent cells of *Escherichia coli* K-12 W3110 (*tyrA*), yielding transformant W3110 (*tyrA*)/pACMAB.

Furthermore, the two plasmids pACMAB and pBR-*aroG4* were used to transform W3110 (*tyrA*) yielding transformant W3110 (*tyrA*)/pBR-*aroG4*.pACMAB. The transformant W3110 (*tyrA*)/pBR-*aroG4*.pACMAB was named AJ 12604 strain and deposited in Fermentation Research Institute of the Agency of Industrial Science & Technology of Japan under the deposit number FERM BP-3579.

(3) Production of L-phenylalanine

The transformant AJ 12604, W3110 (*tyrA*)/pACMAB, W3110 (*tyrA*)/pPHAD, W3110 (*tyrA*)/pPHATerm, and W3110 (*tyrA*) described above were cultured at 37°C for 24 hours in L-phenylalanine-producing medium (containing 20 g of glucose, 29.4 g of disodium hydrogen phosphate, 6 g of potassium dihydrogen phosphate, 1 g of sodium chloride, 2 g of ammonium chloride, 10 g of sodium citrate, 0.4 g of sodium glutamate, 3 g of magnesium sulfate heptahydrate, 0.23 g of calcium chloride and 2 mg of thiamine hydrochloride in 1 liter of water). The results are shown in Table 2. Quantitative assay was performed by high performance liquid chromatography. An outstanding improvement in the fermentative production of L-phenylalanine using the AJ 12604 strain was obtained.

Table 2

Strain	Amount of L-phenylalanine
W3110(<i>tyrA</i>)	0.1
W3110 (<i>tyrA</i>)/pACMAB	0.5
W3110 (<i>tyrA</i>)/pPHAD	0.5
W3110 (<i>tyrA</i>)/pPHATerm	0.5
AJ 12604	3.8

Example 4: Production of L-tryptophan by fermentation

- (1) Construction of a plasmid bearing feedback inhibition-released DS

Plasmid pACYC177 (acquired from National Institute of Heredity; ampicillin resistance, 3.6 Kbp) was digested with restriction enzyme *XhoI*. After the digestion site was made blunt ended by Klenow treatment, an *EcoRI* linker was ligated therewith using T4 DNA ligase to obtain the plasmid pACYC177E, in which the *XhoI* site became *EcoRI*. Next, the plasmid pTS-*aroG4* described in Example 1-(2) and 1-(3) above was digested with restriction enzymes *EcoRI* and *HindIII* to obtain the fragment containing *aroG4*. This fragment was ligated with the *EcoRI*- and *HindIII*-digested

pACYC177E using T4 DNA ligase. Competent cells of the AB3257 strain (described in Example 1) was transformed with the reaction mixture. Among the ampicillin-resistant strains grown, a strain in which auxotrophy for each of L-tyrosine, L-phenylalanine and L-tryptophane disappeared was selected, and a plasmid was extracted. Thus, plasmid pACEG4 (5.1 Kbp) was obtained. An EcoRI-EcoRI fragment containing a gene conferring kanamycin resistance (Kanamycin Gene Block; 1.3 Kbp, manufactured by Pharmacia Fine Chemicals) was ligated with the plasmid pACEG4 at the EcoRI site using T4 DNA ligase, thus yielding the plasmid pACKG4 (resistant to ampicillin and kanamycin, 6.4 Kbp). The procedure of the construction of pACKG4 is outlined in Fig. 5.

(2) Construction of Escherichia coli K-12 bearing a feedback inhibition-released DS gene and a tryptophan operon

Competent cells of Escherichia coli K-12 AGX6aroP strain (described in U.S. Patent No. 4,371,614, incorporated herein by reference; deposit number: NRRL B-12264) bearing the plasmid pGX50 harboring a tryptophan operon was transformed with the pACKG4 plasmid described above to obtain Escherichia coli AGX6aroP/pGX50,pACKG4. The genotype of Escherichia coli AGX6aroP strain is tna, trpR+, aroP.

(3) Production of L-tryptophan

The transformant Escherichia coli AGX6aroP/pGX50,pACKG4 and AGX6aroP/pGX50 described above was cultured at 30°C for 72 hours in L-tryptophan-producing medium (containing 40 g of glucose, 15 g of ammonium sulfate, 1 g of potassium monohydrogen phosphate, 1 g of magnesium sulfate heptahydrate, 0.01 g of ferrous sulfate heptahydrate, 0.01 g of manganese chloride tetrahydrate, 2 g of yeast extract and 40 g of calcium carbonate in 1 liter of water, pH 7). The results are shown in Table 3. Quantitative assay of L-tryptophan was performed by high performance liquid chromatography. An outstanding improvement in the fermentative production of L-tryptophan using the AGX-aroP/pGX50,pACKG strain was obtained.

Table 3

Strain	Amount of L-tryptophan Produced (g/l)
AGX6aroP/pGX50	0.15
AGX6aroP/pGX50,pACKG4	0.45

Obviously, numerous modifications and variations of the present invention are possible in light of the above teachings. It is therefore to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described herein.

SEQUENCE LISTING

5 (1) GENERAL INFORMATION:

(i) APPLICANT: Ajinomoto Co., Ltd.

10 (ii) TITLE OF INVENTION: GENES ENCODING FEEDBACK
INHIBITION-RELEASED ENZYMES, PLASMIDS CONTAINING THE
GENES, AND MICROORGANISMS TRANSFORMED WITH THE PLASMIDS
USEFUL IN PROCESSES FOR PREPARING AROMATIC AMINO ACIDS BY
15 FERMENTAT

(iii) NUMBER OF SEQUENCES: 12

20 (2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

25 (A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid

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45

50

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(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

10 (A) ORGANISM: Escherichia coli
(B) STRAIN: K-12 MC1061

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

15 GCTAACCAGT AAAGCCAACA

20

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

20 (A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

25 (ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

30 (A) ORGANISM: Escherichia coli
(B) STRAIN: K-12 MC1061

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

35 CCCACTTCAG CAACCAGTTC

20

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

40 (A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

45 (ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

50

55

(vi) ORIGINAL SOURCE:
 (A) ORGANISM: Escherichia coli
 (B) STRAIN: K-12 MC1061

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:
 GTATTACCC CGTTATTGTC

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
 (A) ORGANISM: Escherichia coli
 (B) STRAIN: K-12 MC1061

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:
 ACTCCGCCCG AAGTGACTAA

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 948 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
 (A) ORGANISM: Brevibacterium lactofermentum
 (B) STRAIN: AJ 12125

(ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 1..945

EP 0 745 671 A2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

5	ATG AGC CAC CCA CCA ATT GTT GTG GCC TAT TTG GGG CCT GCC GGA ACC Met Ser Asp Ala Pro Ile Val Val Ala Tyr Leu Gly Pro Ala Gly Thr	48
	1 5 10 15	
	TTC ACC GAA GAA GCC CTC TAC AAA TTT GCC GAC GCC GGC GTA TTC GGC Phe Thr Glu Glu Ala Leu Tyr Lys Phe Ala Asp Ala Gly Val Phe Gly	96
	20 25 30	
10	GAC GGT GAG ATC GAG CAG CTA CCA GCC AAA TCG CCA CAA GAA GCT GTC Asp Gly Glu Ile Glu Gln Leu Pro Ala Lys Ser Pro Gln Glu Ala Val	144
	35 40 45	
15	GAC GCG GTC CGC CAC GGC ACC GCC CAG TTC GCG GTG GTC GCC ATC GAA Asp Ala Val Arg His Gly Thr Ala Gln Phe Ala Val Val Ala Ile Glu	192
	50 55 60	
	AAC TTC GTC GAC GGC CCC GTC ACC CCC ACC TTC GAC GCC CTT GAC CAG Asn Phe Val Asp Gly Pro Val Thr Pro Thr Phe Asp Ala Leu Asp Gln	240
20	65 70 75 80	
	GGC TCC AAC GTG CAA ATC ATC GCC GAA GAA GAA CTC GAT ATT GCC TTT Gly Ser Asn Val Gln Ile Ile Ala Glu Glu Glu Leu Asp Ile Ala Phe	288
	85 90 95	
25	TCC ATC ATG GTC CGG CCA GGG ACT TCG CTT GCC GAC GTC AAA ACC CTC Ser Ile Met Val Arg Pro Gly Thr Ser Leu Ala Asp Val Lys Thr Leu	336
	100 105 110	
	GCC ACC CAC CCG GTT GGG TAC CAA CAA GTG AAA AAC TGG ATG GCA ACC Ala Thr His Pro Val Gly Tyr Gln Gln Val Lys Asn Trp Met Ala Thr	384
30	115 120 125	
	ACC ATT CCG GAC GCC ATG TAT CTT TCA GCA AGC TCC AAC GGC GCC GGC Thr Ile Pro Asp Ala Met Tyr Leu Ser Ala Ser Ser Asn Gly Ala Gly	432
	130 135 140	
35	GCA CAA ATG GTT GCC GAA GGA ACC GCC GAC GCA GCC GCA GCG CCC TCC Ala Gln Met Val Ala Glu Gly Thr Ala Asp Ala Ala Ala Pro Ser	480
	145 150 155 160	
	CGC GCA GCC GAA CTC TTC GGA CTG GAA CGC CTT GTT GAT GAT GTC GCC Arg Ala Ala Glu Leu Phe Gly Leu Glu Arg Leu Val Asp Asp Val Ala	528
40	165 170 175	
	GAC GTC CGC GGC GCC CGC ACC CGC TTC GTT GCA GTC CAA GCC CAA GCA Asp Val Arg Gly Ala Arg Thr Arg Phe Val Ala Val Gln Ala Gln Ala	576
	180 185 190	
45	GCC GTT TCC GAA CCG ACC GGC CAC GAC CGC ACC TCC GTC ATT TTC TCC Ala Val Ser Glu Pro Thr Gly His Asp Arg Thr Ser Val Ile Phe Ser	624
	195 200 205	

50

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CTA CCG AAT GTG CCA GGC AGC CTC GTG CGC GCC CTC AAC GAA TTC GCC 672
 Leu Pro Asn Val Pro Gly Ser Leu Val Arg Ala Leu Asn Glu Phe Ala
 210 215 220
 5 ATC CGT GGC GTC GAC CTC ACC CGC ATC GAA TCC CGC CCC ACC CGC AAA 720
 Ile Arg Gly Val Asp Leu Thr Arg Ile Glu Ser Arg Pro Thr Arg Lys
 225 230 235 240
 10 GTC TTC GGA ACC TAC CGC TTC CAC CTG GAC ATA TCC GGA CAT ATC CGC 768
 Val Phe Gly Thr Tyr Arg Phe His Leu Asp Ile Ser Gly His Ile Arg
 245 250 255
 GAC ATC CCC GTC GCC GAA GCC CTC CGC GCA CTC CAC CTC CAA GCC GAA 816
 Asp Ile Pro Val Ala Glu Ala Leu Arg Ala Leu His Leu Gln Ala Glu
 260 265 270
 15 GAA CTC GTA TTC GTC GGT TCC TGG CCC TCC AAC CGT GCA GAA GAC AGC 864
 Glu Leu Val Phe Val Gly Ser Trp Pro Ser Asn Arg Ala Glu Asp Ser
 275 280 285
 20 ACG CCC CAA ACC GAC CAA CTA GCT AAC GTA CAC AAG GCG GAC GAA TGG 912
 Thr Pro Gln Thr Asp Gln Leu Ala Asn Val His Lys Ala Asp Glu Trp
 290 295 300
 GTT CGC GCA GCA AGC GAA GGA AGG AAA CTT AAC TAG 948
 Val Arg Ala Ala Ser Glu Gly Arg Lys Leu Asn
 305 310 315

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 315 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

35 Met Ser Asp Ala Pro Ile Val Val Ala Tyr Leu Gly Pro Ala Gly Thr
 1 5 10 15
 Phe Thr Glu Glu Ala Leu Tyr Lys Phe Ala Asp Ala Gly Val Phe Gly
 20 25 30
 40 Asp Gly Glu Ile Glu Gln Leu Pro Ala Lys Ser Pro Gln Glu Ala Val
 35 40 45
 45 Asp Ala Val Arg His Gly Thr Ala Gln Phe Ala Val Val Ala Ile Glu
 50 55 60

Asn Phe Val Asp Gly Pro Val Thr Pro Thr Phe Asp Ala Leu Asp Gln
 65 70 75 80
 5 Gly Ser Asn Val Gln Ile Ile Ala Glu Glu Glu Leu Asp Ile Ala Phe
 85 90 95
 Ser Ile Met Val Arg Pro Gly Thr Ser Leu Ala Asp Val Lys Thr Leu
 100 105 110
 10 Ala Thr His Pro Val Gly Tyr Gln Gln Val Lys Asn Trp Met Ala Thr
 115 120 125
 Thr Ile Pro Asp Ala Met Tyr Leu Ser Ala Ser Ser Asn Gly Ala Gly
 130 135 140
 15 Ala Gln Met Val Ala Glu Gly Thr Ala Asp Ala Ala Ala Pro Ser
 145 150 155 160
 Arg Ala Ala Glu Leu Phe Gly Leu Glu Arg Leu Val Asp Asp Val Ala
 165 170 175
 20 Asp Val Arg Gly Ala Arg Thr Arg Phe Val Ala Val Gln Ala Gln Ala
 180 185 190
 25 Ala Val Ser Glu Pro Thr Gly His Asp Arg Thr Ser Val Ile Phe Ser
 195 200 205
 Leu Pro Asn Val Pro Gly Ser Leu Val Arg Ala Leu Asn Glu Phe Ala
 210 215 220
 30 Ile Arg Gly Val Asp Leu Thr Arg Ile Glu Ser Arg Pro Thr Arg Lys
 225 230 235 240
 Val Phe Gly Thr Tyr Arg Phe His Leu Asp Ile Ser Gly His Ile Arg
 245 250 255
 35 Asp Ile Pro Val Ala Glu Ala Leu Arg Ala Leu His Leu Gln Ala Glu
 260 265 270
 Glu Leu Val Phe Val Gly Ser Trp Pro Ser Asn Arg Ala Glu Asp Ser
 275 280 285
 40 Thr Pro Gln Thr Asp Gln Leu Ala Asn Val His Lys Ala Asp Glu Trp
 290 295 300
 45 Val Arg Ala Ala Ser Glu Gly Arg Lys Leu Asn
 305 310 315

50 (2) INFORMATION FOR SEQ ID NO:7:

(1) SEQUENCE CHARACTERISTICS:

55

(A) LENGTH: 948 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Brevibacterium lactofermentum*
 (B) STRAIN: AJ 12259

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ATGAGCGACGCACCAATTGTTGTGGCCTATTG	60
GGGCGCTGCCGGAACCTTCACCGAAGAA	
GCCCTCTACAAATTTGCCGACGCCGGCGTATT	120
CGGCGACGGTGAGATCGAGCAGCTACCA	
GCCAAATCGCCACAAGAAGCTGTGACGCGGT	180
CCGCCACGGCACCGCCCGAGTTCGCGGTG	
GTCGCCATCGAAAATTCGTCGACGGCCCCGTC	240
ACCCCCACCTTCGACGCCCTTGACCAG	
GGCTCCAACGTGCAATCATCGCCGAAGAAGAA	300
CTCGATATTGCCTTTTCCATCATGGTC	
CGGCCAGGGACTTCGCTTGCCGACGTCAAAAC	360
CCCTCGCCACCCACCGGTTGGGTACCAA	
CAAGTGAAAACTGGATGGCAACCACCATTC	420
CGGACGCCATGTATCTTTCAGCAAGCTCC	
AACGGCGCCGGCGCAAAATGGTTGCCAAGGA	480
ACCGCCGACGCCGAGCCGCGAGCGCCCTCC	
CGCGCAGCCGAACTCTTCGGACTGGAACGC	540
CTTGTTGATGATGTGCGCGACGTCCGCGGC	
GCCCGCACCCGCTTCGTTGCAGTCCAAGCC	600
CAAGCAGCCGTTTCCGAACCGACCGGCCAC	
GACCGCACCTCCGTCATTTCTCCCTACCGA	660
ATGTGCCAGGCAGCCTCGTCCGCGCCCTC	
AACGAATTCGCCATCCGTGGCGTCGACCTC	720
ACCCCGCATCGAATCCCGCCCCACCCGCAA	
GTCTTCGGAACCTACCGCTTCCACCTGGAC	780
ATATCCGGACATCCCGGACATCCCGGTC	
GCCGAAGCCCTCCGCGCACTCCACCTCCA	840
AGCCGAAGAACTCGTATTGTCGGTTCCTGG	
CCCTCCAACCGTGCAAGACAGCAGCCCCAA	900
ACCGACCAACTAGCTAACGTACACAAG	
GCGGACGAATGGGTTCCGCGCAGCAAGCGA	948
AGGAAGGAACTTAAGTAG	

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 315 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Met Ser Asp Ala Pro Ile Val Val Ala Tyr Leu Gly Pro Ala Gly Thr
 1 5 10 15
 Phe Thr Glu Glu Ala Leu Tyr Lys Phe Ala Asp Ala Gly Val Phe Gly
 20 25 30
 Asp Gly Glu Ile Glu Gln Leu Pro Ala Lys Ser Pro Gln Glu Ala Val
 35 40 45
 Asp Ala Val Arg His Gly Thr Ala Gln Phe Ala Val Val Ala Ile Glu
 50 55 60
 Asn Phe Val Asp Gly Pro Val Thr Pro Thr Phe Asp Ala Leu Asp Gln
 65 70 75 80
 Gly Ser Asn Val Gln Ile Ile Ala Glu Glu Glu Leu Asp Ile Ala Phe
 85 90 95
 Ser Ile Met Val Arg Pro Gly Thr Ser Leu Ala Asp Val Lys Thr Leu
 100 105 110
 Ala Thr His Pro Val Gly Tyr Gln Gln Val Lys Asn Trp Met Ala Thr
 115 120 125
 Thr Ile Pro Asp Ala Met Tyr Leu Ser Ala Ser Ser Asn Gly Ala Gly
 130 135 140
 Ala Gln Met Val Ala Glu Gly Thr Ala Asp Ala Ala Ala Pro Ser
 145 150 155 160
 Arg Ala Ala Glu Leu Phe Gly Leu Glu Arg Leu Val Asp Asp Val Ala
 165 170 175
 Asp Val Arg Gly Ala Arg Thr Arg Phe Val Ala Val Gln Ala Gln Ala
 180 185 190
 Ala Val Ser Glu Pro Thr Gly His Asp Arg Thr Ser Val Ile Phe Ser
 195 200 205
 Leu Pro Asn Val Pro Gly Ser Leu Val Arg Ala Leu Asn Glu Phe Ala
 210 215 220
 Ile Arg Gly Val Asp Leu Thr Arg Ile Glu Pro Arg Pro Thr Arg Lys
 225 230 235 240

Val Phe Gly Thr Tyr Arg Phe His Leu Asp Ile Ser Gly His Ile Arg
 245 250 255
 Asp Ile Pro Val Ala Glu Ala Leu Arg Ala Leu His Leu Gln Ala Glu
 260 265 270
 Glu Leu Val Phe Val Gly Ser Trp Pro Ser Asn Arg Ala Glu Asp Ser
 275 280 285
 Thr Pro Gln Thr Asp Gln Leu Ala Asn Val His Lys Ala Asp Glu Trp
 290 295 300
 Val Arg Ala Ala Ser Glu Gly Arg Lys Leu Asn
 305 310 315

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Escherichia coli
 (B) STRAIN: K-12 RR1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TCAACAAGCT GGAACGGACC

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Escherichia coli*
(B) STRAIN: K-12 RR1

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CGCCGATTTA CCGCCTTGAG

20

10 (2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CCGTCTGGAA CCACGCCGA T

21

25 (2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

35 (iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

40 ATCGGGCGTG ATTCCAGACG G

21

45 Claims

1. A DNA fragment containing a mutated *aroF* gene encoding 3-deoxy-D-arabinoheptulonic acid-7-phosphate synthase, in which the amino acid residues in positions 147 and/or 181 are substituted or one or more amino acid residue(s) are deleted or added, and which is released from feedback inhibition.
2. A DNA fragment containing a mutated *aroG* gene encoding 3-deoxy-D-arabinoheptulonic acid-7-phosphate synthase, in which one or more amino acid residues in positions 146, 147, 150, 157, 202, 219, and 332 are substituted or one or more amino acid residue(s) are deleted or added, and which is released from feedback inhibition.
3. A DNA fragment according to claim 1, in which the aspartate residue 147 is substituted by an asparagine and the serine residue 181 is substituted by a phenylalanine.
4. A DNA fragment according to claim 2, in which the substituent of the aspartate residue 146 is an asparagine residue, the substituent of the methionine residue 147 is an isoleucine residue, the substituent of the proline residue

150 is a leucine residue, the substituent of the methionine residue 157 is an isoleucine residue, the substituent of the alanine residue 202 is a threonine residue, the substituent of the alanine residue 219 is a threonine residue, and the substituent of the glutamate residue in position 332 is a lysine residue.

- 5 5. A vector, in which the DNA fragment according to any of claims 1 to 4 is operably linked to regulatory DNA effecting expression of said protein encoding DNA.
6. A microorganism comprising a vector according to claim 5.
- 10 7. A microorganism, in which the DNA fragment according to any of claims 1 to 4 is integrated into the chromosome and is operably linked to regulatory DNA effecting expression of said protein encoding DNA.
8. A microorganism according to claim 6 or 7, which additionally comprises a mutated gene encoding chorismate mutase-prephenate-dehydratase from *Escherichia coli* or prephenate-dehydratase from a *Corynebacterium* bacterium,
 - 15 wherein one or two amino acid residue(s) are substituted or one or more amino acid residue(s) are deleted.
9. A microorganism according to claim 8, wherein the serine residue in position 330 of chorismate mutase-prephenate-dehydratase is substituted or amino acid residues downstream from the 330 serine residue are deleted or
 - 20 wherein the serine residue in position 235 of prephenate-dehydratase is substituted.
10. A microorganism according to claim 9, wherein said serine residues are substituted by a proline residue or an aspartate residue.
- 25 11. A process for preparing an aromatic amino acid which comprises culturing a microorganism claimed in any of claims 6 to 10 in a medium and isolating the aromatic amino acid produced.
12. A process for preparing an amino acid as claimed in claim 11, wherein said aromatic amino acid is L-phenylalanine or L-tryptophan.

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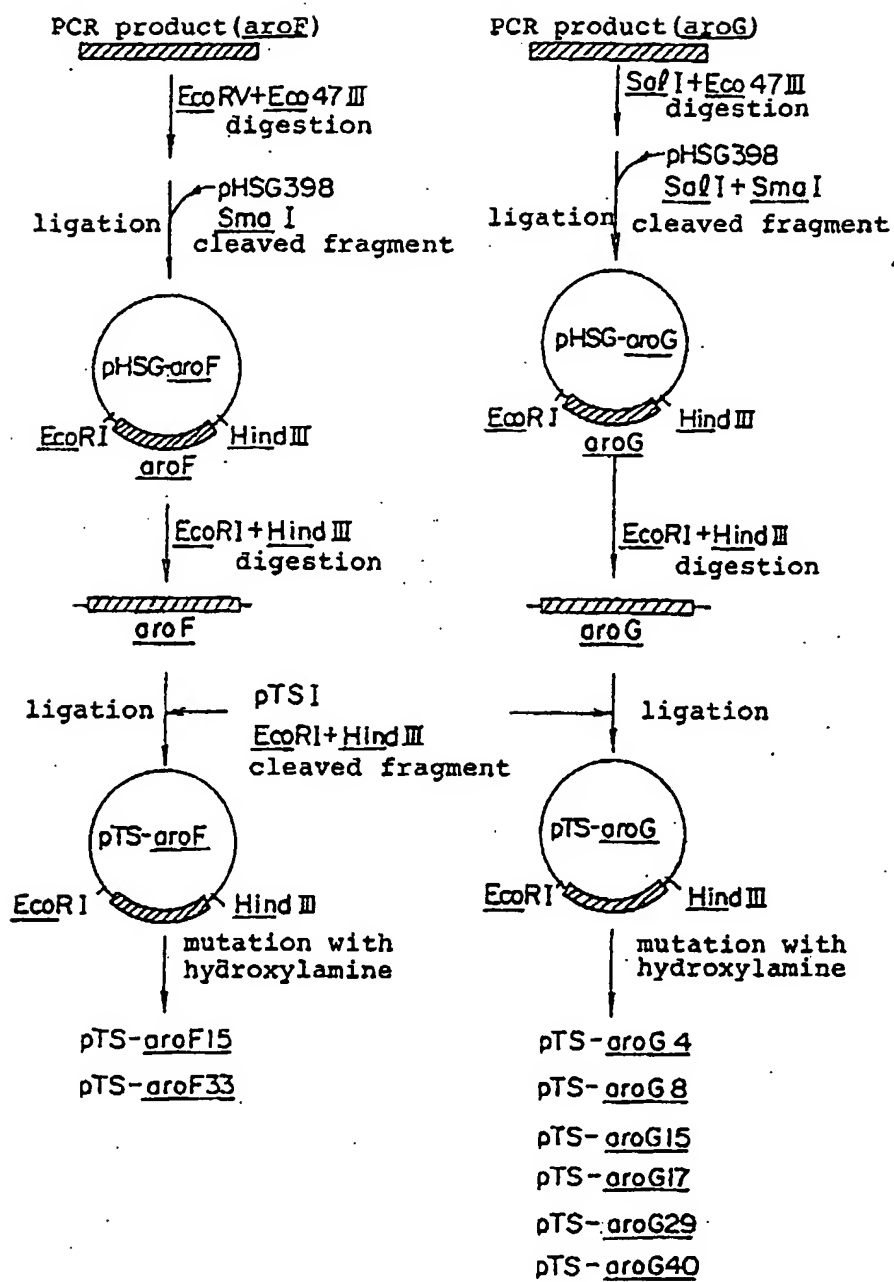
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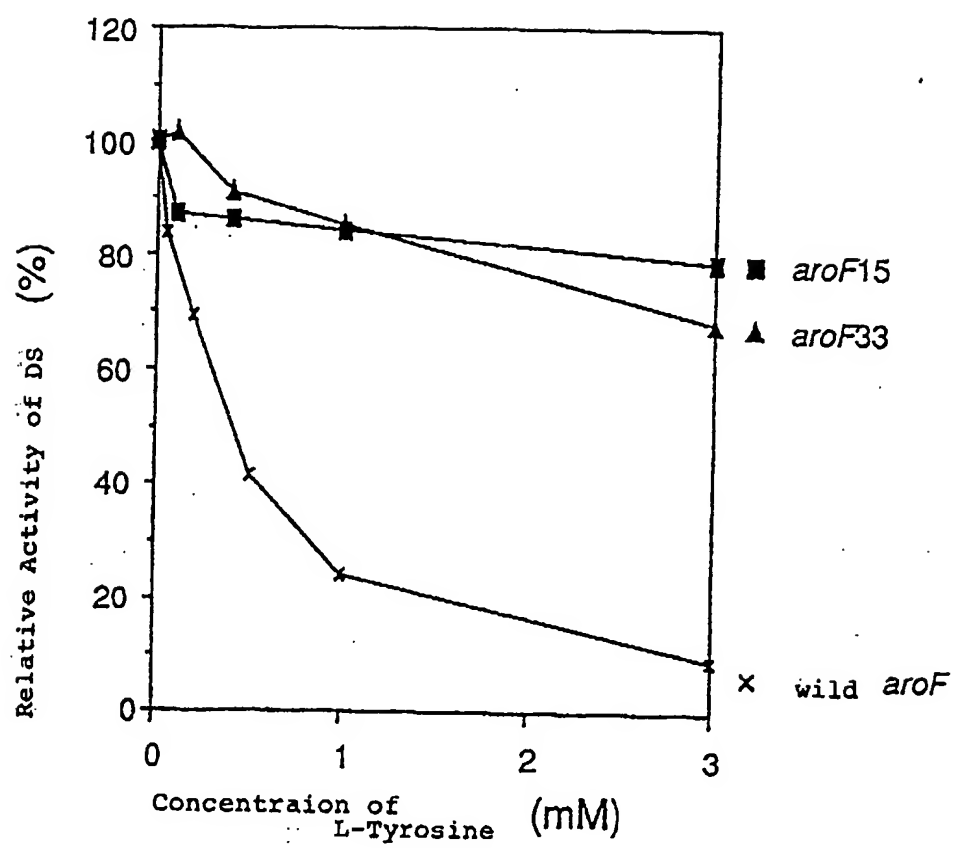
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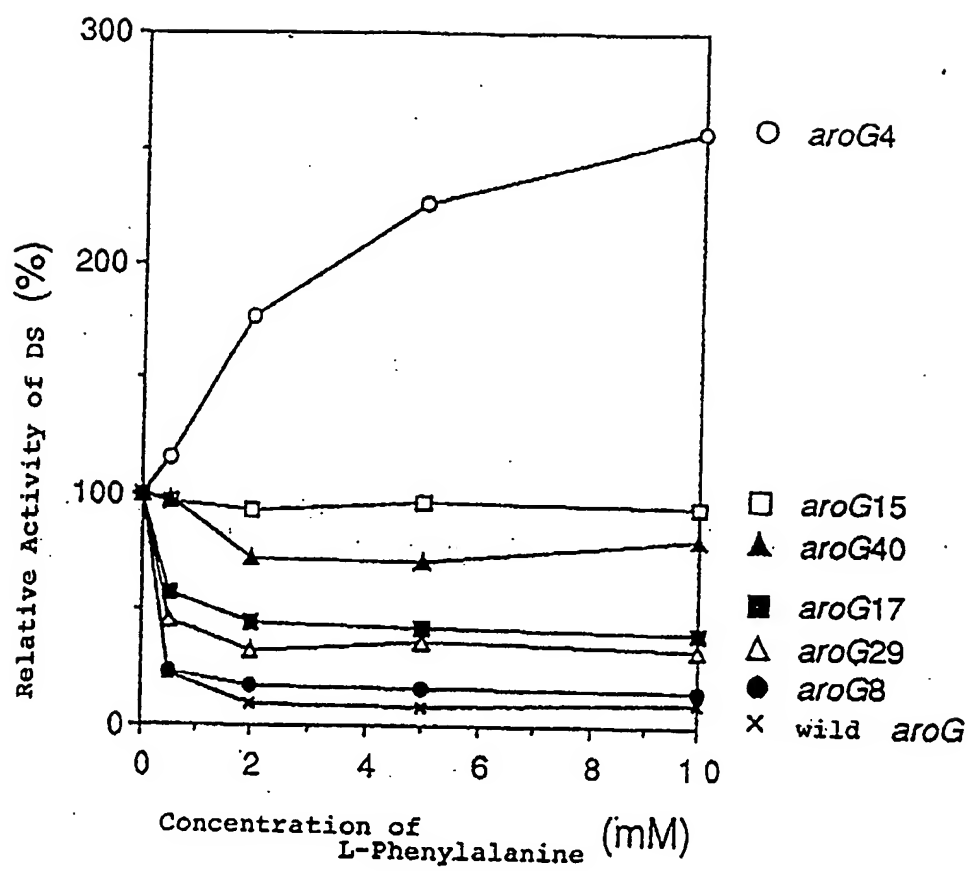
(FIG. 1)



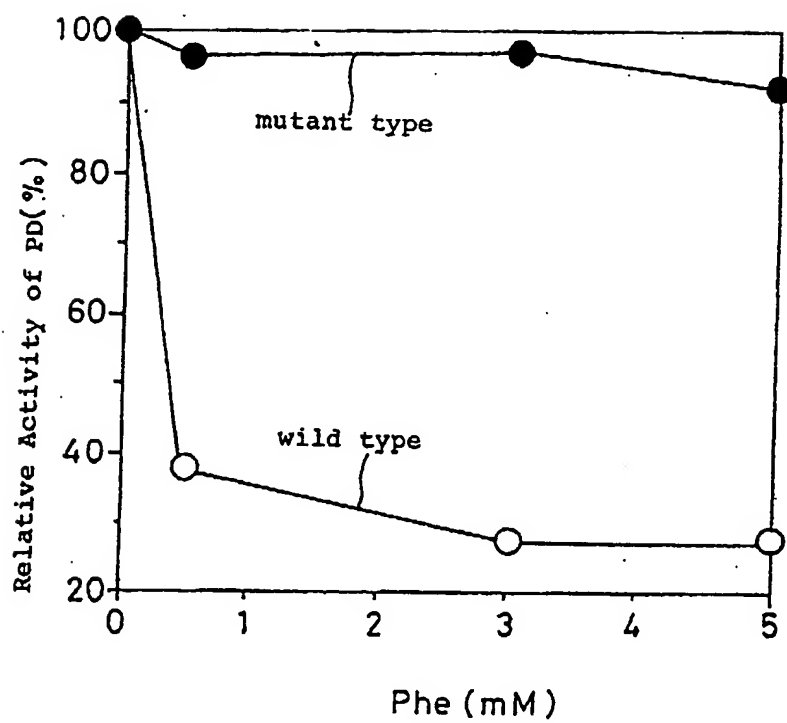
(FIG. 2)



(FIG. 3)



(FIG. 4)



(FIG. 5)

